TEMPERATURE AND THE REGULATION OF ENZYME ACTIVITY IN THE HIBERNATOR. A KINETIC AND SPECTROSCOPIC STUDY OF MUSCLE PYRUVATE KINASE FROM THE ARCTIC GROUND SQUIRREL

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TEMPERATURE AND THE REGULATION OF ENZYME ACTIVITY IN THE HIBERNATOR. A KINETIC AND SPECTROSCOPIC STUDY OF MUSCLE PYRUVATE KINASE FROM THE ARCTIC GROUND SQUIRREL

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ABSTRACT

The properties of pyruvate kinase from skeletal muscle of the Arctic ground squirrel were examined over the annual physiological temperature range of the animal. Successive purified enzymes appeared to be identical in terms of a near-neutral isoelectric point of 6.92 and a molecular weight of 234,000 daltons. Evidence for subunit interaction during inhibition by L-phenylalanine was demonstrated with ultra-violet derivative spectroscopy. A model for this interaction and its importance for a regulatory role is discussed. The absence of a temperature-break in the Arrhenius plot for the pyruvate kinase reaction, the kinetic and physical data, and the near-neutral isoelectric point suggest, when compared to data on the same enzyme from other mammals, a unique amino acid composition that conserves the overall geometry and resultant kinetic behavior which renders the enzyme insensitive to temperature.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The effects of temperature on species subjected to widely varying temperatures have long been of interest to biochemists and physiologists. Enzymes usually have a temperature optimum ie: the temperature at which the enzyme shows maximum catalytic efficiency. If an animal naturally experiences widely changing environmental temperature, then its enzymes must adapt and perform effectively to meet the cellular needs at the new temperature. This research deals in part with the mechanisms of adaptation to temperature.

Enzyme Structure

The enzyme - Nature's Catalyst - is a specialized protein that facilitates reactions under physiological conditions. In such a molecular entity there are four levels of structure to consider; primary, secondary, tertiary, and quaternary structures.

The primary structure is the linear sequence of the component L-α- amino acids and the location of disulfide bridges formed from two cysteine residues. The amino acids are linked by the covalent amide bond between the α-carboxyl group of one amino acid residue and the α- amino group of the adjacent residue in what is known as the peptide bond.

The steric arrangement of neighboring amino acids is termed the secondary structure and involves periodic structures such as the α-helix and the β-pleated sheet. At this level of structure hydrogen

bonds are formed between carbonyl oxygen and an amino hydrogen on a neighboring amino acid.

Tertiary structure of a protein molecule involves the steric relationship of amino acids within a peptide chain that are some distance away in the linear sequence. The dividing line between tertiary and secondary structure is somewhat ambiguous and often arbitrary.

If an enzyme contains several individual polypeptide chains, each of which is called a subunit, then the arrangement of the subunits with respect to one another determines the quaternary structure. This level of structure involves hydrogen bonds, electrostatic interactions, hydrophobic interactions, and van der Waals interactions.

Temperatures that are experienced physiologically do not affect covalent bonds and thus the primary structure of an enzyme will remain stable toward environmental temperatures normally encountered. However, hydrogen bonding, electrostatic interactions, van der Waals interactions, and hydrophobic bonding are the weaker interactions and thus sensitive to temperature. They must be suitably maintained but flexible enough to permit the enzyme to regulate metabolism effectively.

Enzyme Catalysis

Enzymes are important because they allow reactions to occur at high rates with minimal concentrations of substrates at low temp-

erature and also since they are able to regulate metabolism very effectively according to needs of the cell in response to its environment. This section deals with the enzyme as a catalyst and the selective pressures to which it is exposed.

In the following discussion, the concentration of enzyme is negligible compared to that of the substrate, a reasonable assumption due to the high catalytic efficiency of enzymes. Consider the following simple scheme where E is the free enzyme and S the substrate, ES the enzyme-substrate complex, and P the product. If the reaction rate

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

measured is fairly constant over a short period of time, then the concentration of ES, [ES], will be at a steady-state level and thus the steady-state approximation can be applied to [ES] (Briggs & Haldane, 1925). This is done below:

$$\frac{d[ES]}{dt} = 0 = k_1[E][S] - k_2[ES] - k_{-1}[ES] \qquad \text{eqn (1)}$$

Since ES is constant over the interval for measuring the velocity, then the velocity, v, equals k_2 times the concentration of the enzyme substrate complex:

$$v = k_2[ES] \qquad \text{eqn (2)}$$

The concentration of free enzyme, [E], plus the [ES] is the total concentration of enzyme [E_T]. Thus:

$$[E] = [E_T] - [ES] \qquad \text{eqn (3)}$$

Substituting eqn (3) and rearranging eqn (1) yields:

$$k_1([E_T] - [ES])[S] - k_2[ES] - k_{-1}[ES] = 0$$

$$[ES] = [E_T][S]/([S] + (k_2 + k_{-1})/k_1 \qquad \text{eqn (4)}$$

and from eqn (2):

$$v = k_2[ES] = k_2[E_T][S]/([S] + (k_2 + k_{-1})/k_1) \qquad \text{eqn (5)}$$

This equation is identical to the Michaelis-Menten equation (Michaelis & Menten, 1913) where k_2 is sometimes called the turnover number (kcat) which is the number of substrate molecules converted to products per unit time per active site and $(k_2 + k_{-1})/k_1$ is equal to Km, the Michaelis-Menten constant. If the enzyme is saturated with substrate, then [ES] is a maximum and the product of k_2 and [E_T] is the maximum velocity of the reaction (Vmax). When the velocity is one half Vmax, then Km = [S], which is shown in the following scheme:

$$Vmax = kcat[E_T]$$

$$v = kcat[E_T][S]/([S] + Km) = \tfrac{1}{2}Vmax = Vmax[S]/([S] + Km)$$

$$[S] + Km = 2[S] \qquad Km = [S]$$

Km is then said to be the substrate concentration at one half maximal velocity. If kcat (k_2 in this scheme)$\ll k_{-1}$, then $Km = k_{-1}/k_1 = Ks$, the enzyme-substrate dissociation constant. This approximation assumes that the enzyme-substrate complex is in thermodynamic equilibrium with free enzyme and substrate. In this instance, Km is a measure of the enzyme-substrate affinity, and a large Km is interpreted to mean a low affinity of the enzyme for substrate. Nature appears to have appreciated this relationship at an early stage because modulation of enzyme-substrate affinity is a powerful method of regulating enzyme activity.

Meaning of kcat

The turnover number (kcat) is the maximum number of substrate molecules converted to products per active site per unit time. It thus has units of a first order rate constant ($time^{-1}$). In the simple Michaelis-Menten scheme, eqn (1), kcat is simply the first order rate constant for conversion of the ES complex to E + P which is the chemical step in the reaction scheme. In more complex reactions where

more than one enzyme intermediate exists, kcat is a function of all the first order rate constants. Consider the scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P_2$$
$$\qquad\qquad\qquad\qquad\quad \downarrow P_1$$

where EA is an intermediate, P_1 is the first product and P_2 the second. By applying the steady-state assumption to [EA], that is, the change in [EA] with time is zero, the following rate equation can be derived:

$$v = [E_o][S]\left(\frac{k_2k_3/(k_2 + k_3)}{K_Sk_3/(k_2 + k_3) + [S]}\right) \qquad \text{eqn (6)}$$

$$\text{where } Km = K_Sk_3/(k_2 + k_3) \qquad \text{eqn (7)}$$

$$\text{and } kcat = k_2k_3/(k_2 + k_3) \qquad \text{eqn (8)}$$

In eqn (8), k_2 and k_3 are both first-order rate constants. If one of them is rate limiting, for example $k_2 \ll k_3$, then it becomes kcat. Thus kcat sets a lower limit on the chemical rate constant and cannot be greater than any first-order rate constant (Fersht, 1977).

Notice in eqn (7) above that $Km < K_s$. If $k_2 \ll k_3$, then $Km = K_s$. Thus if $Km < K_s$, it is an indication of intermediates that form after the ES complex.

Meaning of kcat/Km

When [S] Km, the Michaelis-Menten equation reduces as follows:

$$v = \frac{kcat[E][S]}{K_m} \qquad \text{eqn (9)}$$

As eqn (9) shows, kcat/Km is the apparent second-order rate constant and is important in that it relates the reaction velocity to the concentration of free enzyme rather than the [ES]. This is so since at very low substrate concentrations, the enzyme is present mostly as the free enzyme. The lower limit on the second-order rate constant for association of enzyme and substrate is thus set by kcat/Km and this term also determines the specificity of the enzyme for competing substrates (Fersht, 1977).

Enzyme Regulation

The breakdown of food sources for the production of energy and the basic building blocks for cells is accomplished by a complex of inter-dependent reaction sequences known as metabolic pathways. Each individual reaction has an enzyme that is highly specific for its substrate with the product of one enzyme the substrate for the next. By regulating the enzymes and their reaction velocities, metabolism is then also effectively controlled, resulting in a balanced supply of both chemical energy and building blocks.

Within a given metabolic sequence, certain enzymes act as control

points for the pathway and are usually located at the beginning and branch points of the metabolic network. These enzymes are known as regulatory enzymes and usually have the following characteristics:

1) the enzyme is oligomeric (multiple subunits).

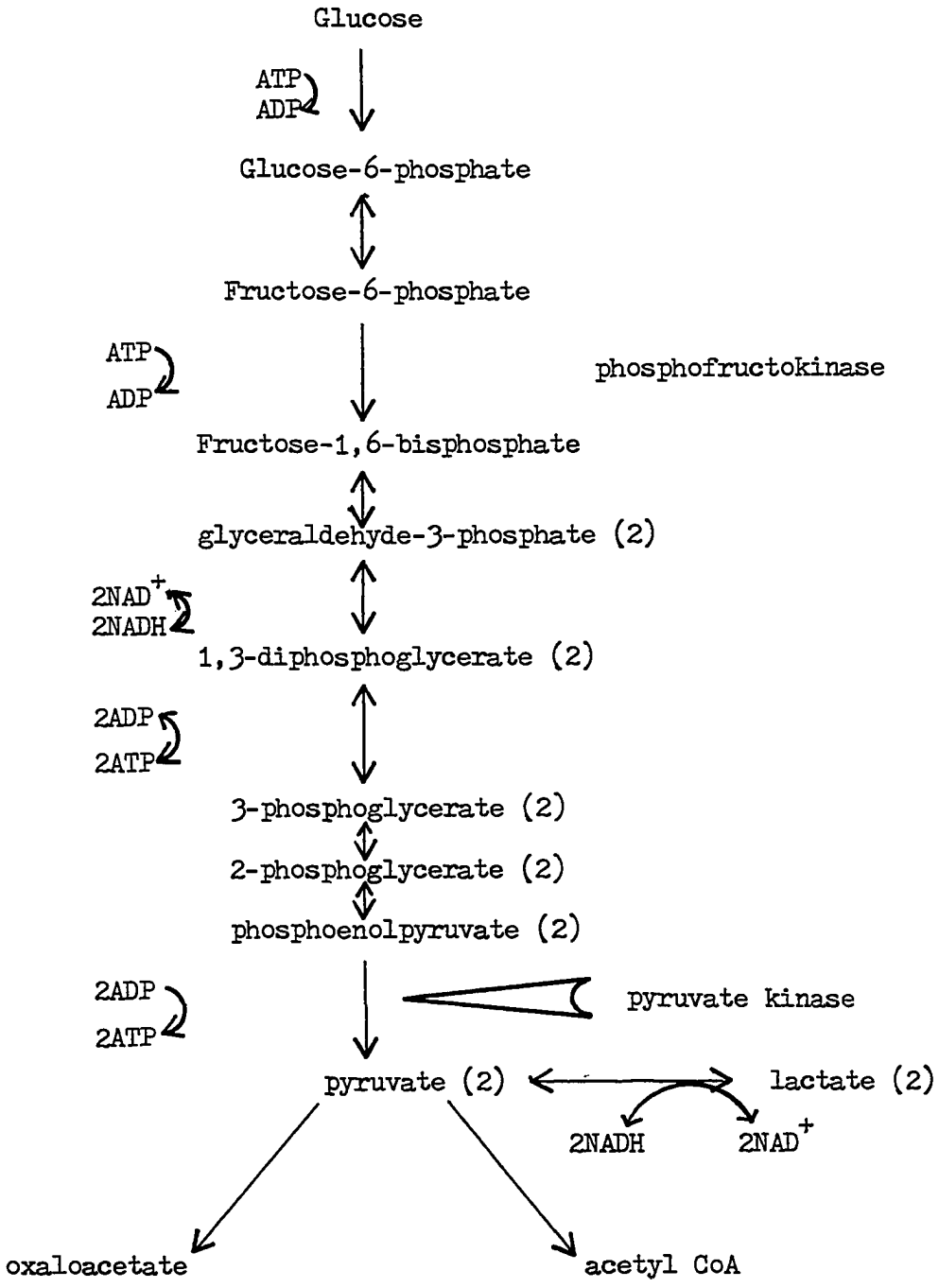
2) a plot of reaction velocity versus substrate concentration results in a sigmoid curve rather that a hyperbolic curve due to subunit interaction upon substrate binding, an interaction known as cooperative-substrate binding.

3) the subunits usually have distinct catalytic and regulatory sites.

4) the enzyme catalyses a reaction that is unidirectional due to the large negative free energy change ($\Delta G^{o'}$).

An excellent example of a regulatory enzyme is pyruvate kinase (PyK) from mammalian liver. This enzyme is a key enzyme for the control of glycolysis (see Fig. 1). Pyruvate, the product of the reaction catalysed by pyruvate kinase has a number of metabolic fates. The enzyme is thus located strategically at a branch point in metabolism. The enzyme has a large $-\Delta G^{o}$ of 31.3 kJ/mole (Lehninger, 1975). The enzyme is oligomeric with four subunits and exhibits cooperative binding for one of its substrates phosphoenolpyruvate (PEP). It shows both product inhibition and feed-forward activation. Adenosine-5'-triphosphate (ATP), a product of glycolysis and also the immediate product of this reaction, inhibits the enzyme. Fructose-1,6-bisphosphate (FBP), a product of an earlier reaction in the same sequence as PyK, activates PyK when binding to a regulatory subunit by

Fig. 1. Glycolysis



lowering the Km for PEP and converting the reaction curve for PEP from sigmoidal to hyperbolic. Thus when glycolysis is turned on and FBP is produced, PyK action is accelerated to avoid a buildup of intermediate substrates. As ATP levels increase PyK activity diminishes resulting in reduced production of ATP. Thus in this pathway, the cell meets its needs for chemical energy (ATP) in a highly self-adjusting or homeostatic manner.

Mechanisms of Temperature Adaptation

Whatever mechanisms for enzymatic temperature adaptation do exist, certain properties must be conserved. First, the higher orders of structure are suitably maintained; secondly, the catalytic properties are adjusted to the needs of the cell at the new temperature; and thirdly, the proper ligand-binding abilities are maintained for the initiation and regulation of catalysis (Somero, 1978). Thus structure, rate, and regulation are the targets of selection in adaptation to temperature.

Three mechanisms of adaptation to wide temperature variations have been suggested: 1) a change in the concentration of intracellular enzymes; 2) enzyme synthesis with different catalytic efficiency; and 3) a change in the intracellular milieu (Behrisch, 1973) . All three appear to operate to some degree, and it is likely that several methods are operating simultanously in concert.

The first scheme, although known to occur with a change in diet (Schimke, 1962) or hormonal stimulation (Weber et al., 1965), is

unlikely to be a major means of temperature adaptation due to the finite solvent capacity of water. In most studies in which enzyme concentration is said to be altered, the enzyme's activity, not its concentration, is actually measured. However, the similar electrophoretic patterns of the warm and cold acclimated enzymes coupled with the time course of changes in activity are consistent with a change in steady-state concentrations. Thus temperature-effected changes are a result of both protein degradation and synthesis, both of which must be finely regulated (see Hazel & Prosser, 1974).

Affinity of an enzyme for its ligands has been shown to be sensitive to temperature in endothermic systems (Helmreich & Cori, 1964). It has therefore been argued that it markedly affects the regulation of metabolism. The extension has been made to ectotherms also. In order for a regulatory enzyme to control metabolism effectively, the Km should lie in a substrate concentration range where changes in Km can effect significant changes in reaction velocity (Cornish-Bowden, 1976). If the substrate concentration is much greater than Km, the enzyme is operating at or near Vmax, while at concentrations of substrate much below Km, the enzyme is barely operating. Thus if Km is a measure of the substrate concentration, a decrease in Km with decreasing temperature permits the enzyme to retain activity at lower (physiological) substrate concentrations (Atkinson, 1969). In contrast if Km does not decrease with temperature Km values at temperatures below the temperature for the minimum Km are so high as to prevent enzyme saturation in vivo.

Animals which experience a change in climatic temperature have bypassed this potential metabolic block in order to survive. Seasonal variants of an enzyme are sometimes formed in response to varying temperature, each variant with the minimum Km near the acclimation temperature. These variants are called isoenzymes or isozymes. Examples are found in muscle fructose bisphosphatase of the Alaskan king crab (Behrisch, 1971), brain acetylcholinesterase in the rainbow trout (Baldwin & Hochachka, 1970), lactate dehydrogenase from yellowfin sole, Limanda aspera, (Behrisch, 1972), and liver pyruvate kinase from the Arctic ground squirrel, Citellus undulatus, (Behrisch, 1974). Variants may also exist between tissues. Shaklee it al. (1977) have studied the qualitative (isoenzymes) and quantitative (activity) characteristics of 12 enzymes from 5 different tissues of green sunfish, Lepomis cyanellus, after thermal acclimation at 2 temperatures, 5°C and 25°C. They noted significant changes in the level of activity of fructose-bisphosphate aldolase, pyruvate kinase, lactate dehydrogenase, malate dehydrogenase, succinate dehydrogenase, cytochrome oxidase, cytochrome C, and glucose-6-phosphate dehydrogenase in one or more tissues. Enzymes in a single metabolic pathway were noted to exhibit parallel changes in a given tissue. However, different tissues did not necessarily give parallel responses, nor were the responses of different pathways similar. Some enzymes in a given pathway increased in activity in response to cold while others in a different pathway decreased demonstrating a major metabolic reorganization. Only a few of the esterases in the liver and

eye exhibited isozymes in Shaklee's study. Thus in the green sunfish, isozyme formation does not play a major role in thermal adaptation. It must be mentioned however, that the green sunfish is a diploid species and the rainbow trout, Salmo gairdneri, in which temperature-specific isozymes coded by duplicated gene loci were discovered, is a tetraploid (Baldwin & Hochachka, 1970). Searches for other examples of this type of adaptation have proved unfruitful in nontetraploid ectotherms capable of tolerating widely varying temperatures (Somero, 1978).

A large literature on the role of intracellular milieu in regulation of enzyme activity exists. Lactate dehydrogenase from skeletal muscle of the yellowfin sole, Limanda aspera, which lives in the Bering Sea year around, provides an excellent example (Behrisch, 1972). A plot of Km vs temperature for this enzyme in the absence of K^+ results in a U-shaped curve with a minimum Km value at about 3°C. The Km shifts to a minimum at 0°C and remains constant to -2°C with the addition of K^+. Since the water temperature varies from a low of -2°C to a high of 4-5°C (Johnson & Hartmann, 1971), this clearly illustrates how a change in cation concentration could render the enzyme activity constant over the physiological temperature range experienced. That such changes in cation concentration and compartmentation do occur in the cell during thermal acclimation has been observed (Rao, 1962, 1967; Hickman et al., 1964; Houston et al., 1970; Umminger, 1969, 1970; Behrisch & Hochachka, 1969a,b; Behrisch, 1969, 1971).

Temperature can also alter the pH of the intracellular milieu, which may in turn influence the net charge of the amino acid side chains in an enzyme and its resultant catalytic activity. In fact the intracellular pH values from numerous animals have been shown to vary inversely with temperature (Malan et al., 1976; Reeves, 1977). The amino acid histidine has a pK that closely follows the change in intracellular pH with temperature and thus results in a preservation of the histidine charge state (Reeves, 1977). It is interesting to note that several workers have demonstrated a shift in pH optima of an enzyme (the pH required for maximum activity) that closely follows the physiological pH with a change in temperature (Rosenmann et al., 1977; Behrisch & Johnson, 1974; Hoskins & Aleksuik, 1973; Park & Hong, 1976). These results strongly suggest a role played by histidine in the molecule's structure. Histidine would then have the effect of extending the temperature range at which an enzyme can effectively function. This would be useful to an animal that has to adapt to temperature over the short-term.

Statement of the Problem

I chose pyruvate kinase from skeletal muscle of the Arctic ground squirrel as an enzyme that might illustrate an adaptive mechanism toward temperature. Pyruvate kinase from skeletal muscle of many mammalian organisms has been well studied and characterized. In particular the enzyme from muscle of the rabbit, an isothermal animal, has been shown to be conformationally sensitive to temperature (Kayne

& Suelter, 1965). In contrast to the rabbit, the Arctic ground squirrel is isothermal during the summer months but heterothermal during the winter season as described below. A natural change in body temperature such as this animal experiences may provide a clue to mechanisms of temperature adaptation by enzymes so that metabolic regulation may continue efficiently.

The enzyme pyruvate kinase (ATP: pyruvate phosphotransferase, E.C. 2.71.40) in the presence of a bivalent cation catalyzes the transfer of a phosphate group from phosphoenolpyruvate to ADP with the resultant formation of pyruvate and ATP.

At least three electrophoretically distinct forms of pyruvate kinase have been observed in most mammalian species (Hall & Cottam, 1978). These have been designated as the L, M_1, and M_2 isoenzymes, with a fourth found in erythrocytes and designated as the R type (Imamura et al., 1972). The L type is the major component in mammalian liver and has kinetics similar to those of the R variant. The M_2 isoenzyme is a minor component in liver and is widely distributed throughout other tissues, while the M_1 type is the major isoenzyme in the brain, cardiac muscle, and the lone variant in skeletal muscle.

The L isoenzyme shows sigmoidal kinetics with respect to one of the substrates, PEP, is activated by fructose-1,6-bisphosphate (Tanaka et al., 1967; Imamura et al., 1972; Eigenbrodt & Schoner, 1977; Kohl & Cottam, 1977), and is assumed to be a regulatory enzyme, whereas the M_1 isoenzyme shows hyperbolic kinetics with respect to PEP and is

unaffected by FBP (Tanaka et al., 1967; Boyer, 1962; Cardenas et al., 1975). All four variants of PyK display Michaelis-Menten kinetics with respect to the second substrate ADP (Hall & Cottam, 1978). The M_2 variant is similar to the L type in that it is allosteric but shows different kinetic values.

In the liver of the Arctic ground squirrel, Citellus undulatus, only one PyK has been detected, which has properties intermediate between those of the L and M_2 isoenzymes found in other mammalian livers (Behrisch, 1974; Behrisch and Johnson, 1974). This PyK exists as two distinct seasonal isoenzymes in response to the annual temperature regime. In the Arctic ground squirrel the onset of hibernation is accompanied by a gradual reduction of metabolic rate to a low of nearly 1/200th of the normothermic rate (Burlington, 1972; Hannon et al., 1972). During hibernation body temperatures may be the same as those of the hibernaculm, falling to as low as 0°C. The hibernator arouses spontaneously and cyclically, and metabolic rates approach normothermic rates within about 90 minutes. The animal remains in this state without feeding for 12-18 hours before again becoming torpid (Fisher et al., 1967; Kayser, 1961). Although the season of hibernation is about eight months, blood glucose concentrations must be maintained within fairly narrow limits (Twente & Twente, 1967). As glucose, the primary substrate for the central nervous system (Cahill, 1970), can be stored in only limited quantities by liver and other tissues, gluconeogenesis must continue during hibernation. The synthesis of seasonal isoenzymes, at least in

liver, is a possible means for maintaining homeostasis of glucose reserves in a widely changing external environment (Behrisch, 1973; Baldwin & Hochachka, 1970).

The enzymes from other tissues of the Arctic ground squirrel adapt to a range of temperatures also, at least during the hibernation season. Thus a mechanism to hold regulation of the enzymes of glycolysis insensitive to temperature would seem necessary for the animal to survive the prolonged hypothermia combined with cyclic active periods and normothermic temperatures.

In this paper I examine pyruvate kinase from skeletal muscle of the Arctic ground squirrel. A procedure for its purification is described. The summer and winter variants of the muscle PyK appear to be identical, in contrast to those of liver. Evidence for subunit interactions during inhibition by an inhibitor, L-phenylalanine, is demonstrated with UV derivative spectroscopy. A model for this interaction and the importance of subunit interaction for a regulatory role of pyruvate kinase from skeletal muscle is discussed. Finally, the combined kinetic, chemical and physical data on this enzyme suggest unique characteristics in amino acid composition that would conserve the geometry and resultant kinetic behavior and, in contrast to other PyK's studied, render the enzyme's regulation independent of temperature.

MATERIALS AND METHODS

Animals

Arctic ground squirrels, Citellus undulatus, were captured in the Alaska Range near Mt. McKinley Park in early and late summer. The animals were fed natural foods, Purina Lab Chow, and water ad libitum. During the summer-active period the animals were kept at 18°C with a seasonal lignt cycle. During the winter-hibernation period the temperature was maintained at 5°C with no light.

Materials

Substrates and Type III lactate dehydrogenase from bovine heart were obtained from Sigma Chemical Company. Bio-Gel A 0.5m, CM Bio-Gel A, and Bio-Gel P-6 were purchased from Bio-Rad Laboratories. All other chemicals were of standard reagent quality.

The enzyme was isolated from thigh muscle (quadriceps femoris). Muscle samples were excised within 10 minutes after death and frozen in liquid nitrogen. The tissue was then stored at -80°C until use.

Enzyme Assays

Pyruvate kinase activity was measured by following the decrease in absorbance at 340 nm using the lactate dehydrogenase coupled reaction (Bucher & Pfleiderer, 1955). Temperature was controlled by means of a circulating water bath and, unless otherwise stated, all assays were carried out at 25°C in 2.98 ml, containing 0.05M

imidazole-HCl buffer, pH 7.00, 100 mM KCl, 10 mM $MgCl_2$, 3 mM ADP, 1mM PEP, 0.15 mM NADH, 5 units LDH, and the reaction was started with addition of enzyme. Enzyme activity was directly proportional to concentration of enzyme and substrates were added at the appropiate buffer pH. Each kinetic experiment was performed in triplicate with less than 10% variation between individual values. Kinetic constants were determined by the weighted least squares method of Wilkinson (1961). One unit of activity is defined as one micromole of pyruvate formed per minute.

Protein Determination

The method of Bradford (1976) was used to measure protein concentrations, and purified rabbit muscle pyruvate kinase (Sigma Chemical Company, St Louis, MO) was used to establish a standard curve. The results of the standare curve were checked against the published $E_{280}^{0.1\%}$ of 0.54 $cm^2 mg^{-1}$ (Boyer, P.D, 1962).

Substrate Concentration

The concentration of PEP was determined enzymatically using excess PyK and assay conditions described above. The reaction was allowed to proceed until a constant A_{340} reading was obtained. The decrease in NADH was compared to a blank using the same assay conditions but omitting the PyK. The concentrations of fresh solutions of ADP were determined spectrophotometrically at 259 nm with ε equal to 15.4 mM^{-1} cm^{-1} (Block et al., 1956).

Enzyme Purification

All steps were carried out at 4°C.

Step 1: Preparation of crude extract. Muscle homogenate was prepared in 3 parts (w/v) of 0.04M imidazole-HCl, pH 7.40 containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The mixture (47.1 g muscle) was stirred in a Sorvall Omni-mixer at high speed for 1 minute and then centrifuged for 20 minutes at 30,000 x g.

Step 2: Ammonium sulfate precipitation. The pellet from step 1 was discarded and the supernatant brought to 40% saturation with the slow addition of solid $(NH_4)_2SO_4$. After 2 hours of stirring the mixture was centrifuged at 30,000 x g for 20 minutes, the pellet was discarded and the supernatant was brought to 80% $(NH_4)_2SO_4$ saturation. After 2 hours of stirring the mixture was centrifuged at 30,000 x g for 20 minutes. The pellet was redissolved in a minimal volume of 0.05 M BIS-TRIS-HCL, pH 6.00 with 10 mM 2-mercaptoethanol and 1 mM EDTA.

Step 3: Gel-filtration chromatography. The above preparation (16 ml, specific activity of 11.6 units/mg protein) was applied to a Bio-Gel A 0.5m column (2 cm x 100 cm) previously equilibrated with 0.05 M BIS-TRIS-HCL, pH 6.00 with 10 mM 2-mercaptoethanol, and 1mM EDTA with a flow rate of 34 ml/hr. This step also served to remove the residual $(NH_4)_2SO_4$. Fractions were collected and assayed for PyK activity. Fractions with 50% of the maximum activity or greater were pooled (34.6 ml) and concentrated by Millipore's immersible-CX molecular separator kit to 19.5 ml. The pooled fractions were noticably turbid

and had a yellowish tinge.

Step 4: CM Bio-Gel A chromatography. The concentrated PyK from step 3 (19 ml) was then centrifuged at 30,000 x g for 20 minutes to remove the turbidity. The supernatant (18.7 ml, specific activity 33.5 units/mg protein) was then applied to a CM Bio-Gel A column (2.0 x 60 cm) previously equilibrated with 0.02 M BIS-TRIS-HCL, pH 6.00 containing 10 mM 2-mercaptoethanol and 1 mM EDTA using a flow rate of 33 ml/hr. A linear ionic strenght gradient, 0-0.3 M KCl, was used to elute the enzyme. Fractions containing PyK activity were pooled (26.8 ml total) and concentrated to 11.2 ml. The resultant preparation was stable for several weeks at 4°C. Freezing resulted in a gradual loss of activity; however, the enzyme could be stored indefinitely as an $(NH_4)_2SO_4$ pellet. Fresh preparations were redissolved from $(NH_4)_2SO_4$ pellets in a minimal amount of buffer and desalted on a Bio-Gel P-6 column.

Isolectrofocusing and Electrophoresis

Isolectrofocusing was carried out at 4°C using the LKB 8100 ampholine electrofocusing equipment according to the manufacturer's recommendations. A typical run lasted 36 hours. Individual fractions, about 2 ml each, were collected and assayed for PyK activity, protein concentration was determined, and the pH was measured. Electrophoresis was performed utilizing LKB's flat-bed electrofocusing equipment in ampholine PAG (polyacrylamide gel) plates. The gel was then stained with Coomassie Brilliant Blue R-250 from Sigma and destained according to LKB's instructions.

Molecular Weight Determinations

Molecular weights wer determined by gel filtration on a Bio-Gel A 0.5m column (2.0 x 60 cm) equilibrated with 0.05 M BIS-TRIS-HCL and 10 mM 2-mercaptoethanol. The flow rate was 52.3 ml/hr. In these experiments the following marker proteins were used: beef liver catalase (MW 240,000), rabbit muscle aldolase (MW 158,000), hen egg albumin (MW 45,000), all from Boehringer Mannheim in a kit. Rabbit muscle pyruvate kinase (MW 237,000), and rabbit muscle enolase (MW 84,000) were purchased from Sigma. The log molecular weight was plotted against a distribution coefficient accorking to Laurent and Killander (1964) and the data analyzed by a least squares procedure.

UV Derivative Spectroscopy

UV derivative spectra were obtained using 1 cm cells in a Perkin-Elmer Model 555 UV/VIS recording spectrophotometer with 1st and 2nd derivative electronics. Experimental conditions for sample and reference cells are listed in the Results section. Before all experiments a background correction scan was run to match the cells. After an equal concentration of protein was added to both sample and reference cells, the background was checked by recording the 1st derivative spectra. In all experiments, a flat stable background was achieved. Perturbants were then added to the sample or reference cells and the 1st derivative spectrum was plotted.

THEORY-UV DERIVATIVE SPECTROSCOPY

The application of derivative spectroscopy is relatively new as the electronic differentiation process has only recently been developed. Second derivative spectroscopy has been used to determine the content of phenylalanine, tryptophan, and tyrosine in proteins (Balestrieri et al., 1978) with higher orders of derivative spectroscopy used to monitor changes in conformation of a protein due to temperature (Whitten et al., 1978). I utilized 1st derivative spectroscopy to approximate the widely used technique of difference spectroscopy (Wetlaufer, 1962) for monitoring a change in conformation of the enzyme. If the spectrum of the enzyme has an absorption spectrum from 280-300 nm described by $A(\lambda)$ and a modulator causes a conformational change in the enzyme, thus changing the environment for tryptophan, then a spectral shift $\Delta\lambda$, in the normal absorption spectrum occurs. The new spectrum can then be represented by $A(\lambda - \Delta\lambda)$. This can be expanded into a Taylor series as follows:

$$A(\lambda - \Delta\lambda) = A(\lambda) + \sum_{n=1}^{\infty} \frac{(-\Delta\lambda)^n}{n!} \left(\frac{d^n A}{d\lambda^n}\right)$$

The difference spectrum then becomes:

$$A = A(\lambda) - A(\lambda - \Delta\lambda) = \sum_{n=1} \frac{\quad}{n!} \left(\frac{d^n A}{d\lambda^n}\right)$$ (Donavan et al., 1961)

If $\Delta\lambda$ is small and the higher order terms are small compared to the 1st derivative terms then the difference spectrum can be approximated by the negative slope of the absorption curve multiplied by the spectral shift, $\Delta\lambda$. This approximation is particularly valid at the maximum wavelength in the 1st derivative spectrum, since $d^2A/d\lambda^2$ at this wavelength is zero. Thus the 1st derivative spectrum can be used to approximate the difference spectrum. It should be noted that the wavelength maxima in the 1st derivative spectrum correspond to wavelengths of greatest slope in the absorption spectra and not to wavelengths of maximum absorbance. If upon addition of a modulator to the enzyme a conformational change does not occur, both A and the 1st derivative spectrum will be zero (see Fig. 6 in results). This approach, of course, assumes that the enzyme has tryptophan in its structure and that a change in conformation will alter this amino acid's environment.

Table I. Purification scheme of pyruvate kinase from skeletal muscle from ground squirrel.

Step	mg protein per ml	Protein (mg)	Total act. (umol min^{-1})	Sp. act. (umol $min^{-1} mg^{-1}$)	Yield (%)	Purifn (x-fold)
Crude supernatant	11.2	1410	10700	7.6	100	1
40-80% $(NH_4)_2SO_4$	70.6	1140	13200	11.6	123	1.5
Bio-Gel A 0.5m	16.1	300	9900	33.5	92	4.4
CM-Bio-Gel A	2.5	28	5200	180	48	24.1

RESULTS

Purification

A representative purification is listed in Table 1. This corresponds to the sample described under Materials and Methods. The specific activity was typically 180-200 umoles pyruvate/min mg protein at 25°C and pH 7. A 50% recovery was typical. The following criteria were used to judge enzyme homogeneity: the enzyme from step 4 of Table 1 yielded 1) a single band on electrofocusing in polyacrylamide gels with carrier ampholytes; 2) a single protein peak in electrofocusing; and 3) a single protein peak on gel filtration.

Physical Properties

The molecular weight of the muscle enzyme as determined by gel filtration chromatography is 234,000 ± 4000 daltons, a result similar to that obtained for other mammalian skeletal muscle pyruvate kinases determined in other laboratories by various procedures (Imamura et al., 1972; Eigenbrodt & Schoner, 1977; Cardenas et al., 1975; Flanders et al., 1971; Cottam et al., 1969). This value is comparable to the 243,000 daltons calculated for the summer-active and winter-hibernator isoenzymes from liver of the Arctic ground squirrel (Behrisch, 1974).

Chemical Properties

Repeated electrofocusing of the muscle enzyme from both the

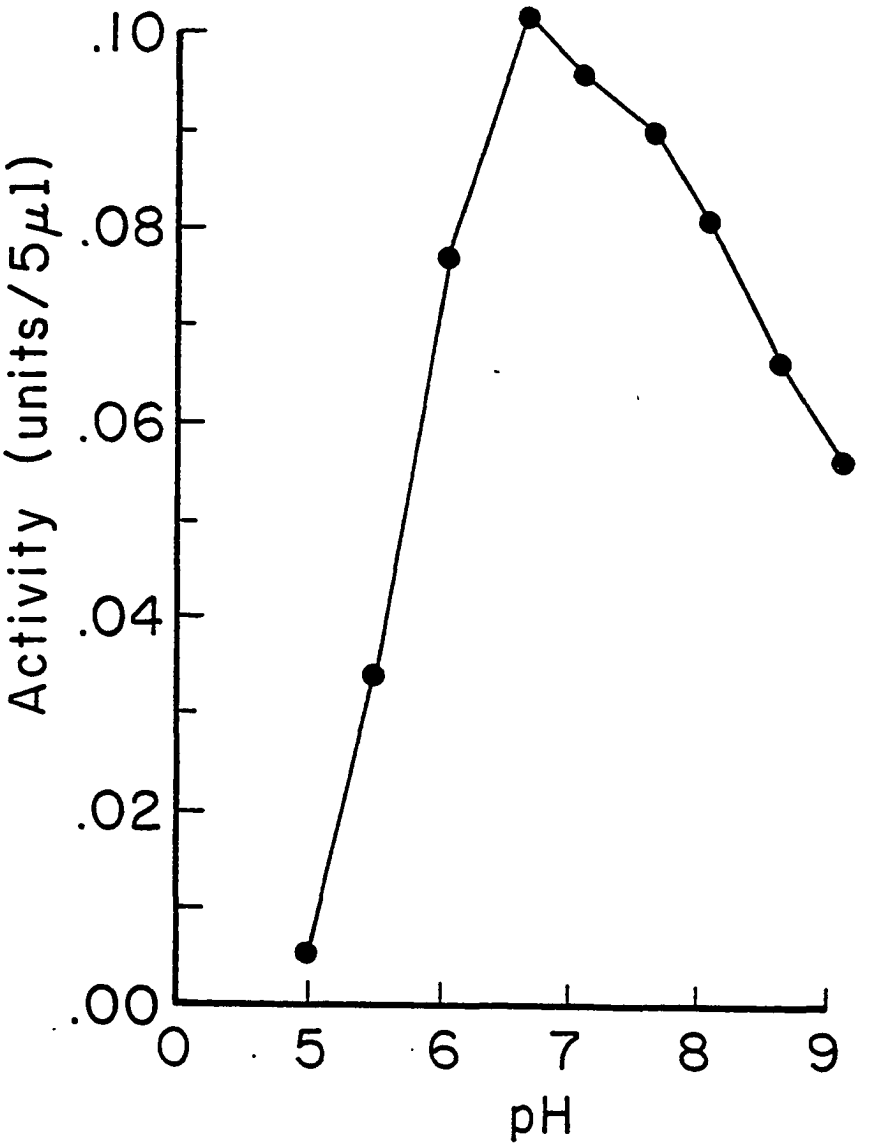


Fig. 2. pH profile of pyruvate kinase from skeletal muscle. Assay conditions at 25°C were 0.05 M imidazole-HCl, 10 mM $MgCl_2$, 1 mM PEP, 1 mM ADP, 0.15 mM NADH, 5 U LDH, and the ionic strength maintained at 0.2 M with KCl.

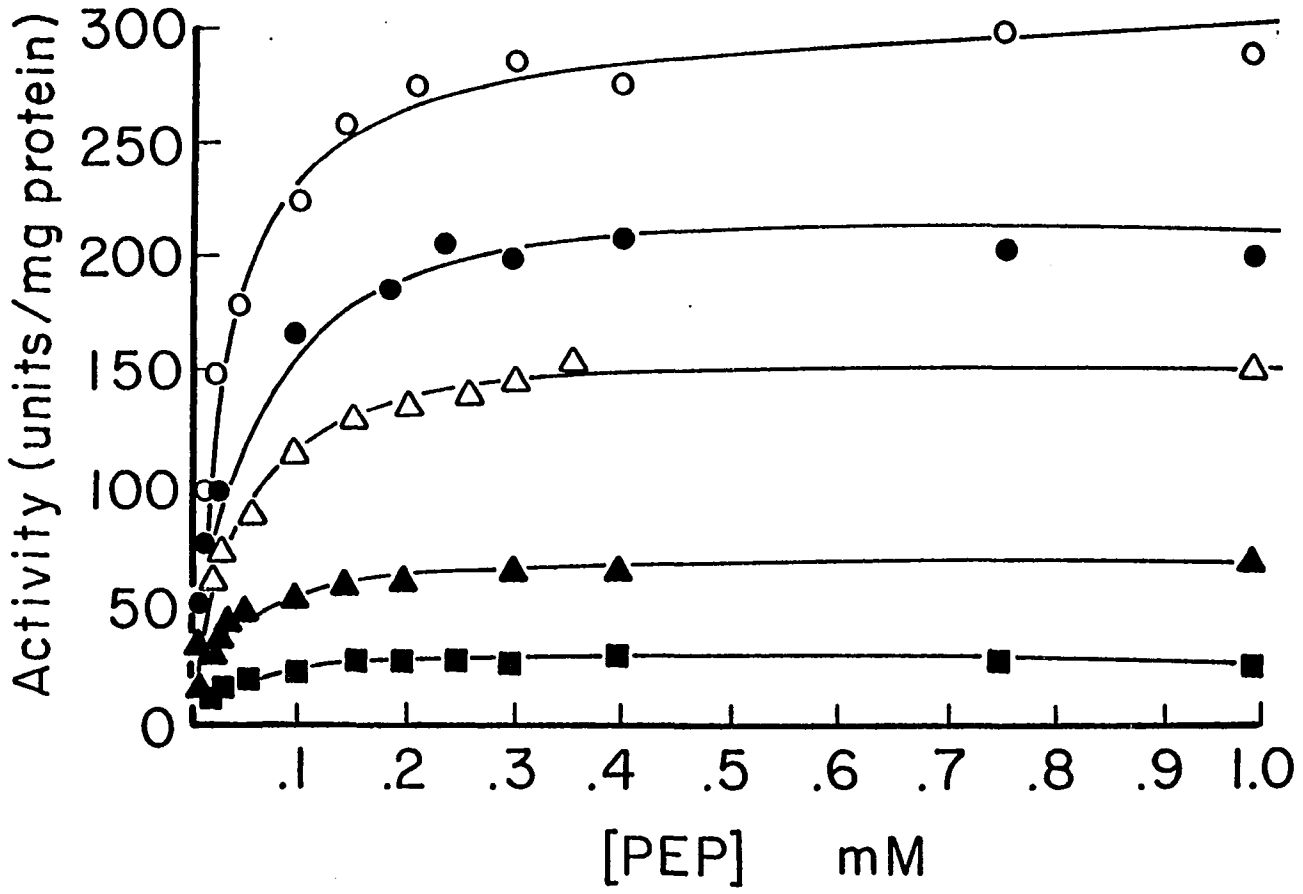


Fig. 3. Enzyme saturation curves for PEP at five temperatures. Assay conditions as in Fig. 2 except as follows: 0.05 M BIS-TRIS-HCl, pH 6.50, and 100 mM KCl. Symbols: opened circles, 35.5°; closed circles, 30.0°; opened triangles, 26.0°; closed triangles, 15.5°; closed squares, 5.0°C.

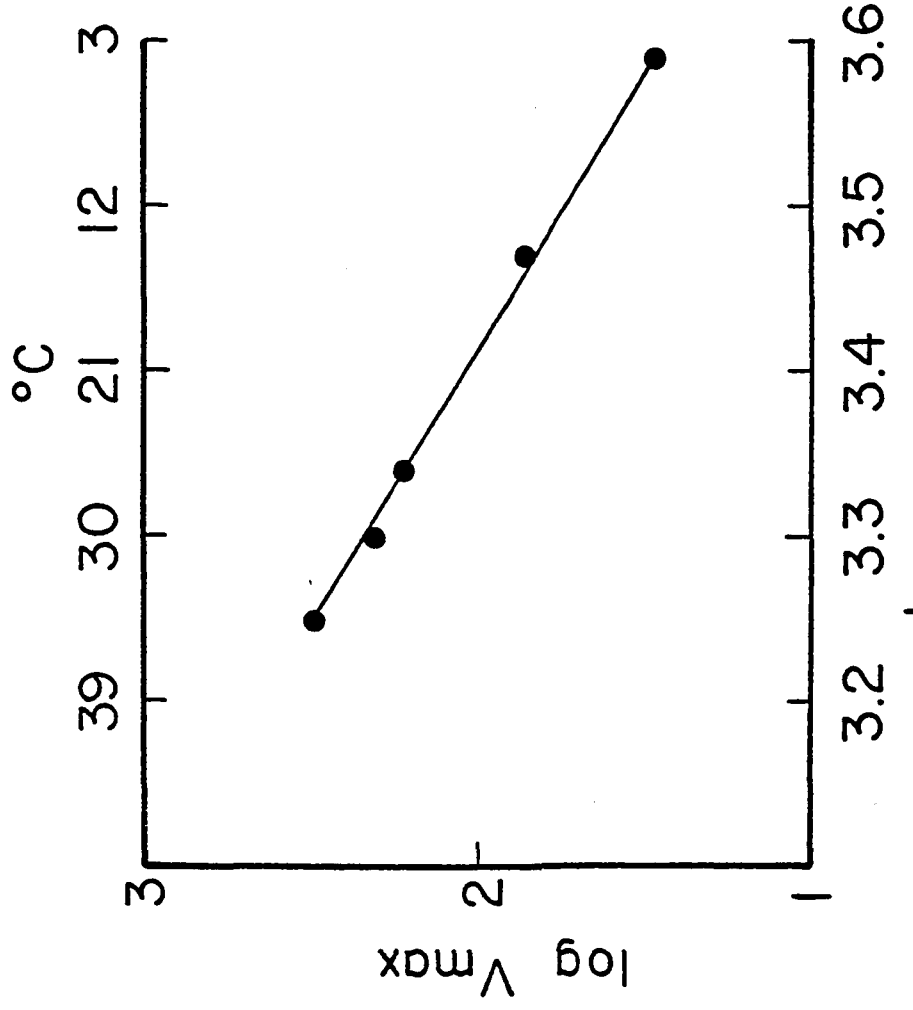


Fig. 4. Arrhenius plot for PyK catalyzed reaction. Vmax was calculated from PEP saturation data of Fig. 3 using the weighted least squares method of Wilkinson (1961).

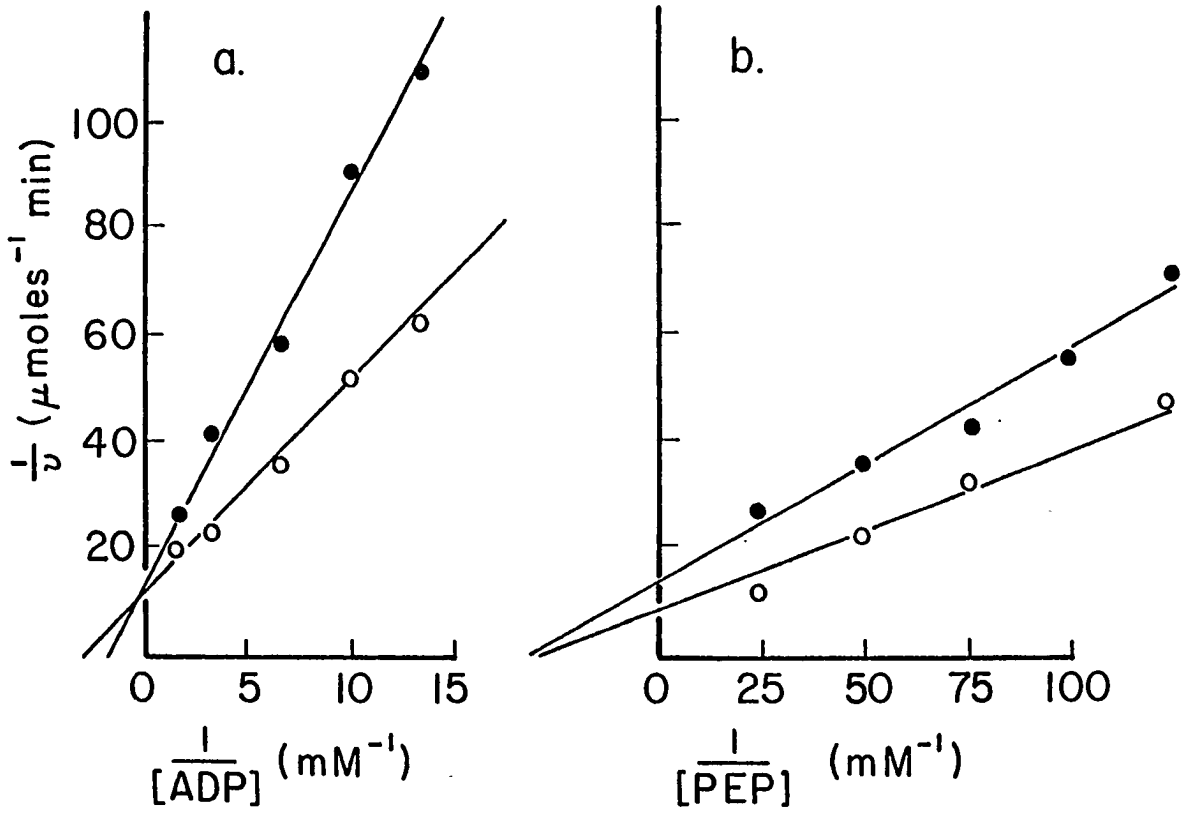


Fig. 5. Double reciprocal plots for the ATP inhibited enzyme. Lines were drawn using the Km and Vmax values calculated by the method of Wilkinson (1961). a) ATP inhibition at varying concentrations of ADP, assay conditions as in Fig. 3. b) ATP inhibition at varying concentrations of PEP, assay conditions as in Fig. 3 except 3 mM ADP. Symbols: closed circles, with ATP; opened circles, without ATP.

hibernator and summer-active squirrel gave an isoelectric point (pI) of 6.92 ± 0.05 pH units, a result dramatically different from those for most other mammalian pyruvate kinases from skeletal muscle eg. pI 8.5-8.9 (Eigenbrodt & Schoner, 1977; Cardenas et al., 1975; Cardenas et al., 1973).

Pyruvate kinase from skeletal muscle of the Arctic ground squirrel has a pH optimum at 6.5 (Fig. 2). Enzyme saturation curves for PEP at five temperatures follow Michaelis-Menten kinetics (Fig. 3). The apparent Michaelis constant, Km, for PEP is a steady 0.03mM throughout the temperature range of 5°-37°C in the presence of 1 mM ADP at pH 6.5. At 1 mM PEP, the apparent Km for ADP, also constant throughout the temperature range studied, is 0.26 mM and follows hyperbolic saturation kinetics. At higher pH (7.40), the apparent Km values for PEP and ADP increase to about 0.08 mM and 0.33 mM respectively.

An Arrhenius plot for the pyruvate kinase reaction is linear over the temperature range studied (Fig. 4). Vmax was calculated from PEP saturation data at each temperature at pH 6.50, using the weighted least squares method of Wilkinson (1961). The activation energy calculated from the slope of this plot is 63.5 kJ/mole, a value similar to that of 65.0 kJ/mole calculated for the high-temperature conformer of rabbit muscle pyruvate kinase (Kayne & Suelter, 1965).

Inhibition of muscle PyK by ATP appears to be competive with respect to ADP and non-competitive with respect to PEP (Fig. 5) with Lineweaver-Burk plots of the data yielding a Ki value of about 1.6 mM.

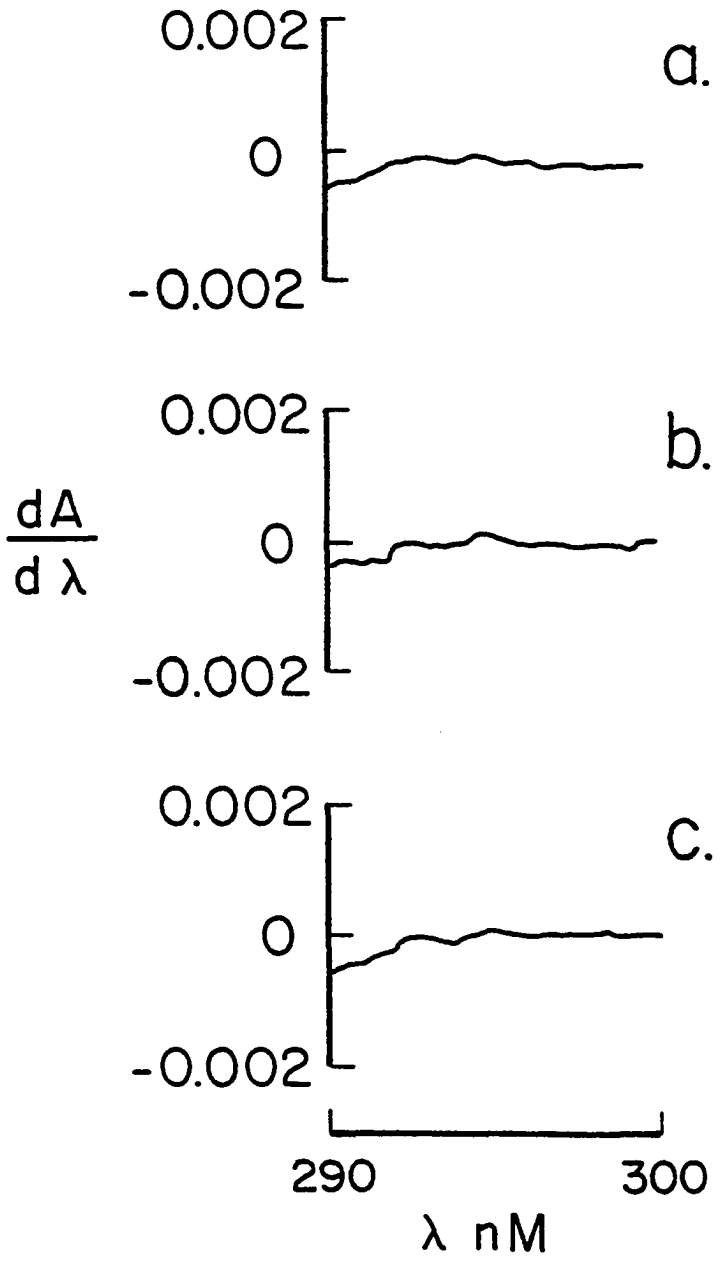


Fig. 6. Derivative spectroscopy of pyruvate kinase in the presence of various modulators. The purified enzyme is 0.75 mg/ml in 50 mM K-HEPES, 100 mM KCl, pH 7.40 at 25°C.

a) PyK + 1 mM ATP vs PyK + 1 mM ADP
b) PyK + 1 mM ATP vs PyK + 1 mM ADP + 5 mM $MgCl_2$
c) PyK + 1 mM ATP + 5 mM $MgCl_2$ vs PyK + 1 mM ADP + 5 mM $MgCl_2$

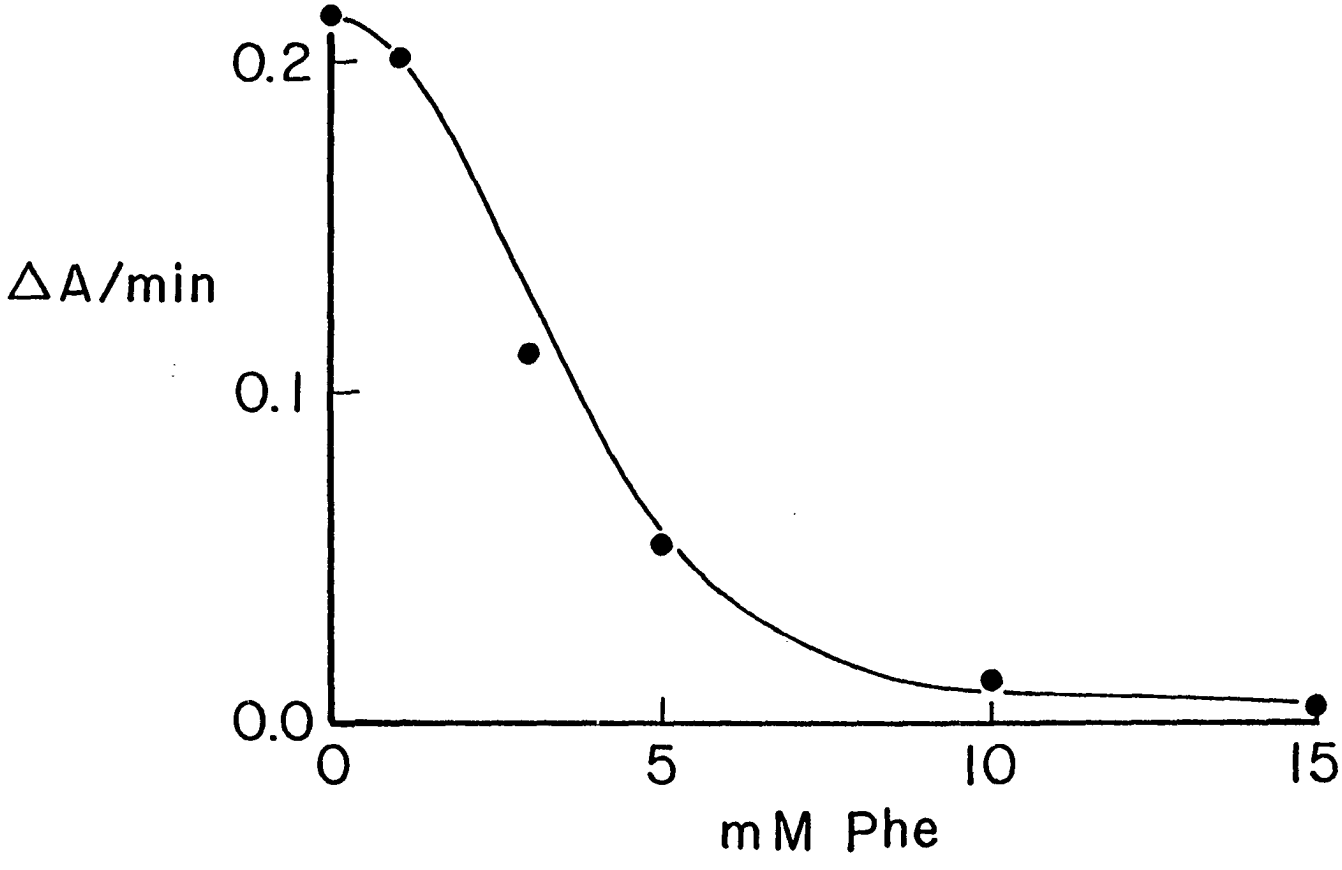


Fig. 7. L-Phenylalanine inhibition. Assay conditions: 0.05 M imidazole-HCl, pH 7.00, 100 mM KCl, 10 mM $MgCl_2$, 0.03 mM PEP, 0.30 mM ADP, 5 U LDH, 0.15 mM NADH, 2 ug enzyme.

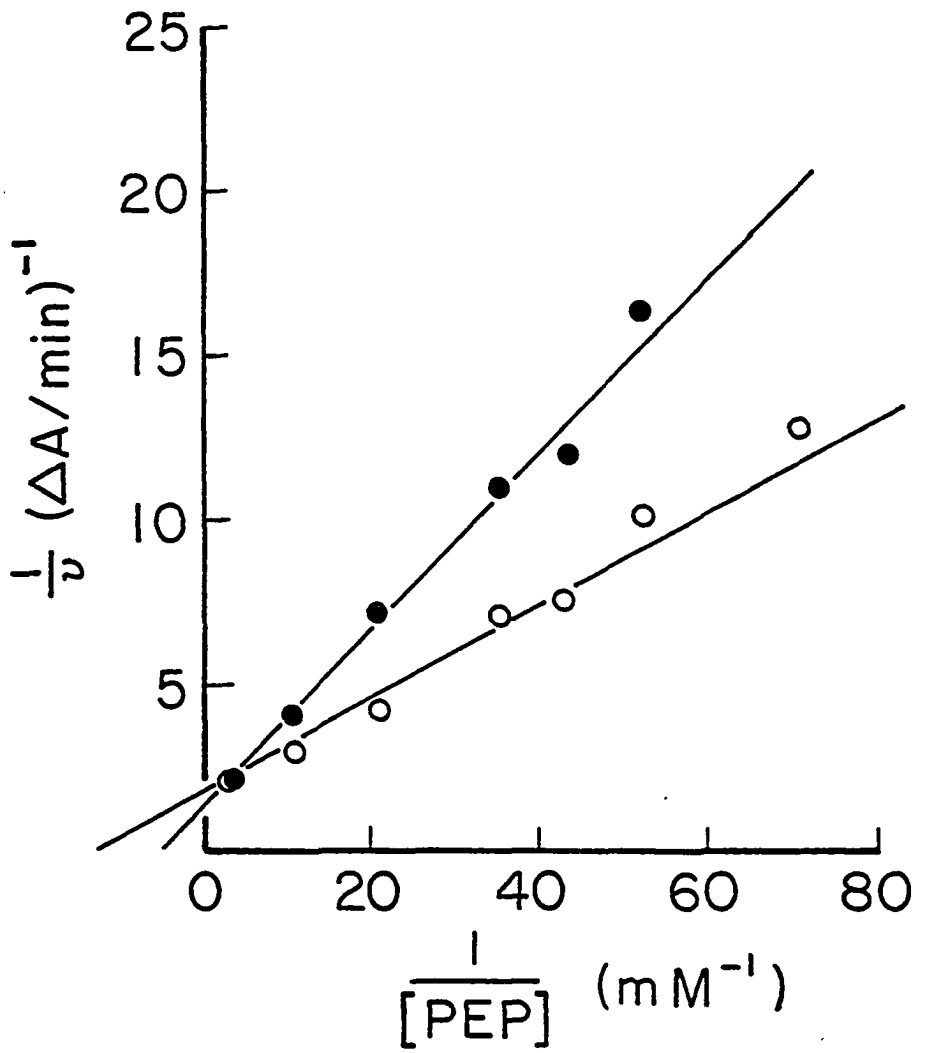


Fig. 8. Double reciprocal plots for PEP saturation with and without L-Phe. Assay conditions: 0.05 M K-HEPES, pH 7.40, 100 mM KCl, 10 mM $MgCl_2$, 3 mM ADP, 5 U LDH, 0.15 mM NADH, 2 ug enzyme, and varying concentrations of PEP. Symbols: closed circles, with 5 mM Phe; opened circles, without Phe.

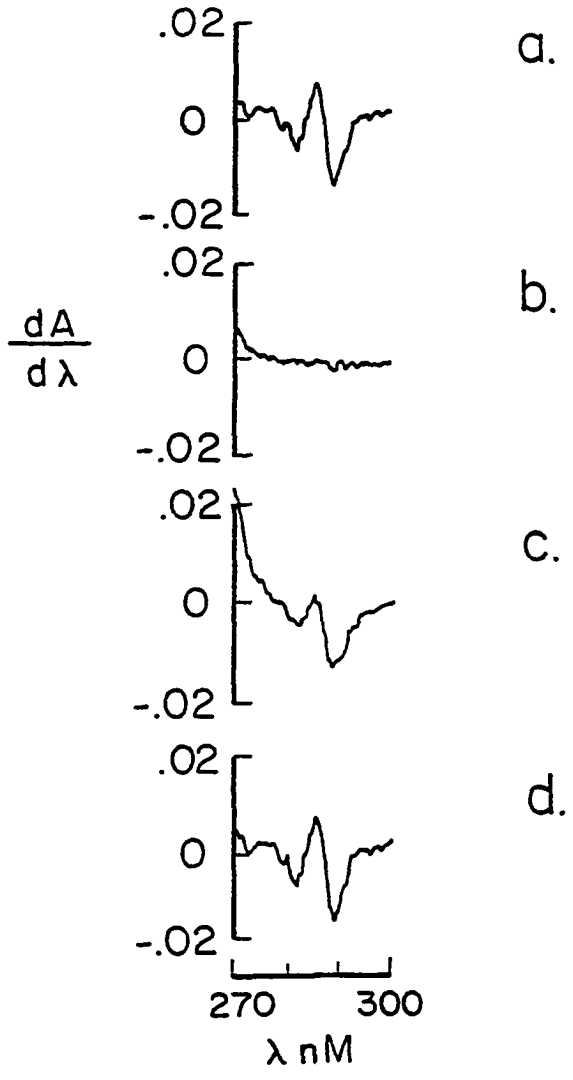


Fig. 9. Derivative spectroscopy for Phe inhibition of PyK. The purified enzyme is 0.75 mg/ml in 50 mM K-HEPES, 100 mM KCl, pH 7.40 at 25°C. a) PyK + 200 uM Phe vs PyK b) PyK + 200 uM Phe + 5 mM $MgCl_2$ vs PyK c) PyK + 1 mM Phe + 5 mM $MgCl_2$ vs PyK d) PyK + 200 uM Phe + 2 mM Ala vs PyK

It appears that the interaction of PyK with ATP in the presence or absence of Mg^{2+} does not effect a change in the enzyme's conformation as seen in the derivative spectra for the ATP-inhibited enzyme (Figs. 6a, b, c). In these experiments it was necessary to keep the ADP and ATP concentrations equal as they absorb very strongly with equal molar absorptivity coefficients at 260 nm.

The amino acid L-phenylalanine (Phe) inhibits the pyruvate kinase catalyzed reaction (Fig. 7). A concentration of 3 mM Phe yields 50% inhibition at pH 7.00 with 0.03 mM PEP and 0.3 mM ADP (Km concentrations of substrates). Another amino acid, L-alanine (Ala), is able to completely reverse the Phe inhibition but has no apparent effect on the enzyme in the absence of Phe. While the inhibition curve for Phe binding is sigmoidal, the degree of site-site cooperativity remains unchanged with Hill constants, nH, for PEP and ADP at 1.03 and 0.98 respectively. Double reciprocal plots for the Phe inhibited reaction demonstrate Phe to be competivite for PEP, with Km for PEP increasing from 0.08 to 0.17 mM while Vmax remains unchanged (Fig. 8). Under saturating conditions of PEP, Vmax and Km for ADP are unchanged. Addition of 200 uM Phe to the enzyme produces a derivative spectrum (Fig. 9a) implying a conformational change affecting the environment of tryptophan. The change produced by 200 uM Phe could be reversed by 5mM $MgCl_2$ (Fig. 9b). Increasing the Phe concentration to 1 mM restored the derivative spectrum (Fig. 9c). Although Ala reversed the inhibition in the kinetic studies, Ala in the absence of Mg^{+2} could not reverse the Phe-induced derivative spectrum (Fig. 9d).

Table II: Summary of properties of muscle pyruvate kinase from the Arctic ground squirrel. For comparative purposes, similar data on pyruvate kinase from ground squirrel liver and rabbit muscle are included.

Parameter	Ground Squirrel Muscle PyK	Rabbit Muscle PyK	Ground Squirrel Liver PyK[a]	
			Summer-Active	Hibernator
Km PEP mM	0.03	0.048[b]	0.95	1.4
Km ADP mM	0.26	0.26[b]	0.130	0.132
Temperature Dependence of Km for PEP and ADP	Independent	Dependent[h]	Dependent	Independent
Isoelectric Point	6.9	8.5-8.9[c]	5.2	5.7
pH Optimum	6.5	7[d]		
Molecular Weight (Daltons)	234,000	237,000[e]	243,000	243,000
Allosteric Effectors				
Ala	No Effect[f]	No Effect[f]	Inhibitory	Inhibitory
Phe	Inhibitory	Inhibitory	Inhibitory	Inhibitory
ATP	Inhibitory[g]	Inhibitory	Inhibitory	Inhibitory
FBP	No Effect	No Effect	Activating	Activating

a. Data are for the enzyme at its acclimation temperature from Behrisch (1974)
b. McQuate and Utter (1959)
c. Range of pI's for most mammalian PyK's from Hall and Cottam (1978)
d. Melchior (1965); Black and Henderson (1972)
e. Warner, R.C. (1958)
f. Reverses Phe inhibition
g. Non-allosteric
h. Low and Somero (1976)

DISCUSSION

The combined kinetic, chemical, and physical data for pyruvate kinase from skeletal muscle of the Arctic ground squirrel are compared to those for pyruvate kinase from rabbit muscle (the properties of which appear to be representative of mammalian muscle PyK) and to those for the seasonal isoenzymes from liver of the Arctic ground squirrel (Table II). It appears that the kinetic properties of muscle PyK's, the M_1 type, are strikingly similar. The apparent Michaelis-constants for PEP and ADP from skeletal muscle of the ground squirrel are nearly identical to those of the rabbit muscle as are other mammalian type M_1 PyK's (Hall & Cottam, 1978). For both animals, the M_1 variant is insensitive to FBP while FBP is a positive effector for the liver variant.

ATP is an inhibitor of muscle PyK, but its mode of action differs from that on the liver enzyme. In the liver PyK of the ground squirrel, ATP acts by dissociating the active enzyme into a less active dimer, a dissociation which can be reversed by FBP (Behrisch, 1974). However, no change occurs in the environment of the tryptophan residues in muscle PyK inhibited by ATP, implying very little or no conformational change (Fig. 6a, b, c). Also gel filtration of the enzyme incubated and eluted with ATP gave a single protein peak with a molecular weight identical to that without ATP. FBP is unable to reverse the ATP inhibition of muscle PyK. These data suggest it is strictly a product inhibition and ATP is not an allosteric inhibitor.

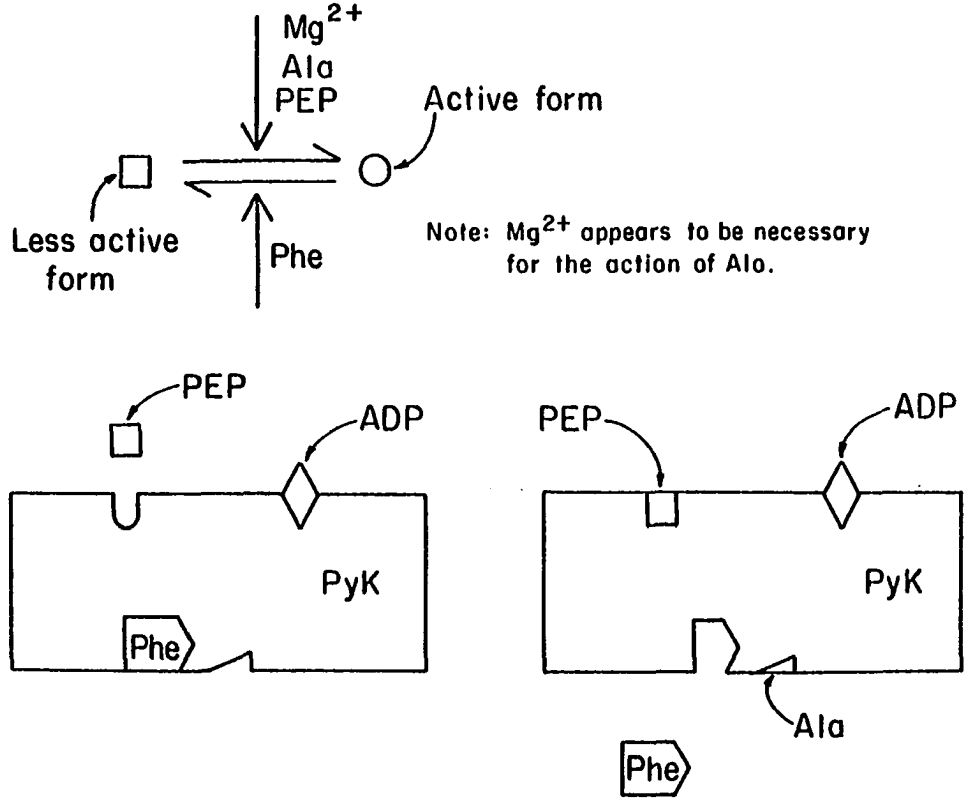


Fig. 10. Schematic model for the L-Phe inhibited enzyme. See text.

As noted previously, the kinetic data revealing ATP to be competive with ADP, and the lack of a conformational change upon ATP-binding imply that ATP and ADP share a common site.

However, the inhibition of muscle PyK by L-Phe appears to be allosteric (see also Carminatti it al, 1971; Kayne & Price, 1972) as suggested by the sigmoid binding kinetics and the 1st derivative spectra. In the absence of L-Ala and Mg^{2+}, 200 uM Phe produces a change in the derivative spectrum from 280-300 nm, a result similar to the difference spectrum for rabbit muscle PyK (Kayne & Price, 1972). This conformational change is completely reversed by Mg^{2+}, but increasing the Phe concentration to 1 mM restores the derivative spectrum. Ala does not reverse this inhibition in the absence of Mg^{2+}. Since Ala can reverse the Phe inhibition in the presence of Mg^{2+}, but by itself has no apparent effect, it is likely that the Phe and PEP sites are separate. This suggests a regulatory role for the muscle PyK which is schematically outlined in Fig. 10. Phenylalanine induces a small conformational change that alters the geometry of the PEP site, but the conformational change is not severe enough to alter the affinity of the enzyme for ADP. Mg^{2+} serves to restore the more active configuration of the enzyme at small concentrations of Phe. Alanine could either compete with the Phe site directly or have a separate site; it alters the affinity of the enzyme for the Phe by inducing a conformational change and thus restores the more active configuration to the enzyme. However, since 2 mM Ala alone did not remove the derivative spectrum produced by 200 uM Phe in the absence

of Mg^{2+} and less than 1 mM Ala completely removes the inhibition by 5 mM Phe in the presence of 10 mM Mg^{2+}, while Mg^{2+} alone will not do this, it appears that the mode of action by Ala requires Mg^{2+}. These results are reminiscent of those of Kayne and Price (1972) who noted in PyK from rabbit muscle that the enhancement of protein fluorescence induced by Phe is not reduced in the absence of PEP or divalent metal cation.

The importance of inhibition of PyK by Phe is a subject of much research and debate (Berglund & Humble, 1979; Tsao, 1979), since Phe inhibition of PyK appears to be a possible mechanism for brain damage in phenylketonuria (Weber, 1969). In their kinetic study of pig muscle PyK Berglund and Humble (1979) concluded that the concentration of Phe needed to inhibit effectively the enzyme in vitro were too high to be of importance in vivo. Tsao (1979) arrived at the same conclusion in a similar study of PyK from the rabbit brain in which type M_1 is a major variant. Those studies as well as the present one show that less than 1 mM Ala can completely reverse the inhibition in vitro. However, I also note that the reversal of inhibition by Ala is dependent upon Mg^{2+}. In the presence of ATP, creatine phosphate and other intermediates of metabolism capable of chelation with Mg^{2+}, the availability of free Mg^{2+} may be reduced enough to limit the action of Ala. Thus, the observed negative allosteric mechanism of Phe inhibition may be of greater importance in the regulation of this enzyme than is currently accepted.

A conspicuous and probable adaptive feature of the PyK from

ground squirrel muscle is the nearly neutral pI (6.9). Judging from the similar kinetic and physical properties, it is likely that the enzyme from the ground squirrel has an overall geometry nearly identical to that of the high temperature conformer of rabbit muscle PyK. However, the break in the Arrhenios plots for PyK from rabbit muscle noted by Kayne and Suelter (1965) may be correlated with conformational changes in the enzyme. No such temperature break occurs in the Arrhenius plot for PyK from ground squirrel muscle from 5° - 37°C (Fig. 4). Judging from the linearity of the Arrhenius plot and from the electrofocusing data, I suggest that the ground squirrel enzyme has been altered by key amino acid substitutions which render the enzyme structurally rigid toward temperature changes in the physiological range yet which conserve the necessary regulatory properties.

CONCLUDING REMARKS AND PROSPECTS

The results of this study, as one might expect, raise more questions than they answer. It appears that the present muscle PyK has evolved an affinity for substrate that is insensitive to temperature. If so, has the conformational flexibility needed for regulation been reduced? Data on PyK from liver (see Table II) show the winter variant of this enzyme to have an affinity for substrate that does not vary with physiological temperature (Behrisch, 1974), a result analogous to that from the muscle enzyme. However, the liver variant exists as a seasonal isoenzyme. Why? Should there be isoenzymes if the winter variant has the necessary conformational flexibility? Or is this the price to be paid for the environmental variability experienced by the ground squirrel during hibernation?

It is interesting to compare liver enzyme with the muscle enzyme. Liver enzyme, activated in the presence of FBP with varying amounts of PEP shows hyperbolic kinetics and a Km value (Behrisch, 1974) similar to that of the muscle enzyme. Is the muscle enzyme merely an activated liver enzyme? By that, I mean, are the binding sites for substrate the same in both enzymes, but some portion of the muscle enzyme (which is a polypeptide) acts like the carbohydrate FBP and thus keeps the enzyme locked in the most active form? Subunit structural studies on the enzymes from liver and skeletal muscle may show some of the subunits to be similar in the two enzymes, and may help to answer this question.

In view of the properties of the enzymes from liver and skeletal muscle, an experiment could be performed in which the subunits of the muscle enzyme are interacted with those of the liver, thus forming a hybrid enzyme. The kinetics of the hybrid could then be measured and compared to those of the native enzymes from muscle and liver. Since both enzymes are tetramers, a number of hybrids can be formed. A hybrid, for example, with one subunit from liver and three from muscle may be expected to have properties largely similar to those of the muscle. Differences may give an insight into the necessity for existence of the two variants M_1 and L and, may also further our understanding of metabolic regulation. The idea is appealing in terms of a metabolic bank account. Identical subunits or portions thereof may represent an expression of a common gene locus which would represent a considerable savings of metabolic machinery and therefore energy.

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